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Time-course study and partial characterization of a protein on hyphae of arbuscular mycorrhizal fungi during active colonization of roots

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Abstract

Material on the surface of hyphal walls of arbuscular mycorrhizal fungi (AMF) during active colonization of plant roots was detected by a monoclonal antibody. Pot-cultured isolates of *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora*, and *Entrophospora* had immunofluorescent material (IM) on younger, thinner, intact hyphae, but IM was scant to absent on thicker, melanized or lysing hyphae. Colonization of corn (*Zea mays* L.), Sudangrass (*Sorghum sudanense* (Piper) Staph.) or red clover (*Trifolium pratense* L.) was examined during 5 months of plant growth by removing cores and performing an indirect immunoassay on roots with attached hyphae. Fresh spores of some *Glomus* spp. had IM on the outer layer of the spore wall. Abundant IM was seen on root hairs of plants colonized by some isolates, and some IM was detected on root surfaces of all plants examined even during early colonization. After cultures were dried, hyphae, roots and spores had little to no IM. Uninoculated control roots had very rare, small patches of IM. An immunoreactive protein was extracted from hyphae of *Gigaspora* and *Glomus* isolates by using 20 m*M* citrate (pH 7.0) at 121 °C for 90 min. Gel electrophoresis profiles indicated that all isolates tested had the same banding patterns. Lectin-binding of extracted protein is suggestive of a glycoprotein. The immunofluorescence assay can be used to examine root sections for active colonization by AMF, and the potential use of the protein to quantify AMF activity in soil is discussed.

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that benefit plants by extending the functional root system and transporting nutrients to plants through hyphae. Hyphae of AMF play an interactive role between the mycorrhiza (fungus and plant roots) and components of the soil and are thought to be important in soil aggregate formation (Miller and Jastrow, 1992). An assay for a fungal product produced by actively-growing hyphae in the soil-plant system may serve as a indicator of soil quality (Wright and Millner, 1994). Immunofluorescence assays (IF) using polydonal antibodies have been developed to detect hyphae of specific AMF (Friese and Allen, 1991; Kough and Linderman, 1986; Wilson et al., 1983). Monoclonal antibody (MAb) technology (Goding, 1986) can be used to select probes that detect antigens common to related organisms.

In this study we report production and testing of an MAb to detect material on extraradical hyphae, root hairs, and roots in the active phase of mycorrhization by isolates of nine species representing all known genera of glomalean fungi except *Sclerosystis*. We present a method to use the antibody in an immunofluorescence assay to help identify and assess the age of AM fungal hyphae over time on plant roots removed from sand. An extraction method for the antigen was developed, and the proteinaceous product is partially characterized by lectin-binding and electrophoretic methods. Indica-

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tions of quantitative differences in protein extracted from different genera are presented.

Materials and methods

Monoclonal antibody production

Freshly collected *Glomus intraradices* FL208 spores were used to immunize a BALB/c mouse. Four intraperitoneal injections of 5,000 spores crushed in 0.5 mL physiological saline solution were made at 3-week intervals and hybridomas were produced (Dazzo and Wright, 1995). Hybridoma 32B11 was selected and cloned based on strong enzyme-linked immunosorbent assay (ELISA) (Wright et al., 1987) reactions with walls and contents of five spores of the targeted isolate, other morphologically similar AMF, and representative isolates of other genera of AMF. Antimouse polyvalent peroxidase-tagged antibody was used to detect the MAb. Antibody 32B11 is an IgM (Sigma Immuno-Type Kit; Sigma Chemical Co., St. Louis, MO). The antibody was produced in bulk by overgrowth of the hybridoma in tissue culture medium amended with thymidine and hypoxanthine (Dazzo and Wright, 1995). Antibody in cell-free supernatant was stored at 4 °C as a sterile solution without preservatives and was used undiluted except where indicated.

Culture conditions and isolates of fungi

Fresh cultures of AMF and control plants (no fungus inoculated) were produced on corn (*Zea mays* L. var. Iochief), Sudangrass (*Sorghum sudanense* (Piper) Staph) or red clover (*Trifolium pratense* L.) using the soilless method described by Millner and Kitt (1992). Plants were grown under greenhouse, growth chamber, or growth room conditions with 18 h of light from sodium vapor lamps (200 to 700 μmol m^{-2} s^{-1}) and a temperature range of 18 to 28 °C until plants senesced (20-22 weeks). Up to four pot culture generations were followed for development of the information presented on some of the AMF.

Fungi from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) tested were: *Acaulospora mellea* Spain and Schenck CL551, *Gigaspora albida* Schenck and Smith FL185 (dubious identity, possibly *Gigaspora rosea*), *Gigaspora gigantea* (Nicol. and Gerd.) Gerdemann and Trappe MA453A, *Gigaspora rosea* Nicholson and Schenck UT102, *Glomus etunicatum* Becker and Gerdemann UT316, *Glomus intraradices* Schenck and Smith FL208, *Glomus intraradices* UT126, and *Scutellospora heterogama* (Nicholson and Schenck) Walker and Sanders BR154C. A culture of *Entrophospora infrequens* (Hall) Ames and Schneider isolated locally from Hagerstown, Maryland was used as a representative of this genus. Corn was the primary test plant, but most isolates also were tested on Sudangrass. Red clover was an alternative host for *Gigaspora gigantea* MA453A, NC110A, and HA115C.

Non-AMF tested for reactivity with MAb 32B11 were *Rhizoctonia solani* Kühn AG2-2 IIIB R^2, *Gaeumannomyces graminis* (Sacc.) Arx and D Olivier var. *avenae* (E M Turner) Dennis, *Gaeumannomyces incrustrans* Landschoot and Jackson, and *Leptosphaeria korrea* J C Walker and A M Smith (courtesy of Henry Wetzel, University of Maryland), *Gliocladium virens* J H Miller, J E Giddens, and A A Foster GL21 and *Endogone pistiformis* Link ex Fries, *Mucor mucedo* P Mich. ex Saint-Amans NRRL 3635. *Zygorhynchus heterogamus* Vuilemin NRRL 1489, *Syncephalastrum racemosum* Con ex J Schröt. NRRL 2496 and *Rhizopus oligosporus* Saito NRRL 2710 (USDA-ARS National Center for Agricultural Utilization Research, Peoria, IL USA); and *Phytophthora megasperma* Drexhs. f. sp. *glycinae* T Kuan and D C Erwin p19 (courtesy Dr M Gallegly, West Virginia University). *E. pisiformis*, *G. virens*, *L. korrea* and the Zygomycetes were cultures on half-strength potato dextrose (PD) broth, and all others were cultured on full or half-strength PD agar.

Immunofluorescence assay

Immunoreactive material (IM) on hyphae attached to roots colonized by AMF isolates was tested by indirect immunofluorescence (IF) over time in individual pot cultures by using a sterile 1.5 or 2.5 cm diameter cork borer to remove a plug of roots. Core material was kept submerged in deionized water in shallow dishes and stored at 4 °C for up to one week. Roots (5-10 cm) with attached extraradical hyphae were removed and gently washed free of sand. Nonmycorrhizal roots were sampled similarly. Samples were processed in fine-mesh sieves in wells of a 12-well tissue culture plate. The following were added in sequence in 1 mL amounts and incubated 1 h: 2% (w/v) non-fat dry milk in phosphate buffered saline (PBS) (Wright, 1994), MAb 32B11, and fluorescein isothiocyanate (FITC)-tagged goat anti-mouse IgM (μ-reactive) diluted in PBS with the addition of 1% (w/v) bovine serum albu-

min (BSA). Following incubation with each antibody, samples were washed extensively with PBS-Tween 20 (PBST) (Wright, 1994). Non-AMF were processed for IF as described above using hyphal mats removed from a broth or agar.

Controls for the antibody reaction were samples processed as described above except that MAb 32B11 was not added, or an IgM antibody reactive with rhizobia was substituted for MAb 32B11. All IF specimens were mounted in Vectashield medium (Vector Laboratories, Burlingame, CA) and viewed on a Nikon Labophot epifluorescence microscope equipped with a HBO 100 mercury lamp, a 460-486 nm excitation filter, a 510 nm dichroic chromatic beam splitter, and a 520-560 nm barrier filter. Fluorescein fluorescence (green) of fungal hyphae and spores was easily distinguished from autofluorescence (yellow) of corn root pieces. Photographs were taken immediately or digitized images were stored for printing later.

Characterization of the antigen on hyphae attached to roots

Root sections colonized by *G. intraradices* UT126 mycorrhizae were subjected to a variety of treatments to characterize the native antigen. Other isolates used for a test or a group of tests are given in parentheses. Lability to heat was determined by immersing roots for 30 min at 100 °C in: (i) water, (ii) 10 m*M* citric acid (pH 6.0), or (iii) PBS at pH 7.4 (*G. gigantea* MA453A). Extraction of the antigen with a detergent was tested by using 2.5% sodium dodecyl sulfate (SDS) at 100 °C for 5 min. Tolerance of pH extremes was tested by incubation in 50 m*M* sodium acetate (pH 4.5) at 37 °C for 8 hours (*G. intraradices* FL208, *G. rosea* UT102, *A. mellea* CL551, and *G. albida* FL185), and in 1 *M* sodium hydroxide (approx. pH 14.0) at 4 °C for 15 h. Controls were removed from the same core as test samples and incubated under the same conditions as test samples except that PBS at pH 7.4 was substituted for test reagents.

The effect of dry storage on the antigen was assessed by performing the IF assay on roots of senesced plants dried in the pots for 4 to 26 weeks at room temperature.

Extraction of the antigen

Extraradical hyphae of *G. gigantea* MA453A, *G. rosea* UT102, *G. intraradices* UT126 and FL208, and *G. etunicatum* UT316 (with numerous adhering spores) was manually picked under the dissecting microscope from colonized roots and potting material and held in water. This material was rapidly blotted dry on laboratory tissue paper and placed in 2-3 mL of extraction buffer (20 m*M*, pH 7.0 citric acid, trisodium salt). Hyphae were ground in a tissue grinder before autodaving for 90 min at 121 °C (Keen and Legrand, 1980). The suspension was centrifuged, and the supernatant was removed and transferred to a centrifugal concentrator (Centricon 10, Amicon, Beverly, MA). Citrate buffer was replaced with water and the sample was concentrated. A Bradford protein analysis (Bio-Rad, Richmond, CA) using BSA as the standard was performed on the concentrated sample. Extract from *G. gigantea* MA453A was further purified by precipitation with 10% (w/v) tricholoracetic acid (TCA). The precipitate was resuspended and washed three times with 20 m*M* TRIS using a Centricon 50. Extracts were held at 4 °C. Air-dry weight of hyphal residue after extraction was recorded. Intact hyphal strands of *G. intraradices* UT126 were tested by IF after extraction to indicate that the procedure had removed the IM.

The hyphal mat from 4-week-old broth-cultured *L. korrea* was extracted with 3 mL of the extraction buffer and the extract was washed and concentrated as described above. Air-dry weight of material remaining after extraction was recorded.

Characterization of extracted protein

Quantitative comparisons of the reaction of protein extracts with MAb 32B11 was by enzyme-linked immunosorbent assay (ELISA). Four replicate wells of a polyvinylchloride assay plate were loaded with 0.5 μg of protein in 50 μL of PBS, dried overnight at 45 °C and blocked with milk (see IF assay) for 15 min. The following incubations, using 50 μL/well were for 1 h each: MAb 32B11 (1:2 dilution in PBS), biotinylated goat anti-mouse IgM (μ-chain reactive), and streptavidin peroxidase. Fifty microliters of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) color developer with the substrate (Wright, 1994) was added and absorbance was read at A_{405} after 15 min. Wells were washed with PBST three times between each reactant. Estimates for concentration of protein used and the dilution of the MAb to give a reading A_{405} = 1.00 were determined by box titration (Wright, 1994) on extracted protein from *G. gigantea* MA453A and *G. intraradices* UT126. Controls for ELISA were a *Rhizobium*-reactive IgM MAb substituted for MAb

32B11 or no primary antibody to test for non-specific adsorption of IgM or endogenous peroxidase.

Reactivity of the extracted protein with the lectins succinyl-concanavalin A (ConA), *Glycine max* agglutinin (SBA), *Lens culinaris* agglutinin (LcH), and *Triticum vulgaris* agglutinin (WGA) was tested by using biotinylated lectins in an microplate ELISA. Extracted protein, 0.125 μg in 50 μL PBS, was adsorbed on polyvinylchloride assay plates by overnight incubation at 45 °C. Blocking buffer was 4% (w/v) BSA and 1% (v/v) Tween-20, and incubation was for 15 min. Fifty microliters containing 2.5 μg lectin diluted in pH 6.8 phosphate buffered saline (with the addition of Ca and Mn to the ConA buffer (Wycoff et al., 1987)) was incubated for 1 h followed by three washes with PBST. Streptavidin peroxidase was added to wells and incubated 1 h followed by four washings with PBST. Color development was as described above using ABTS.

Electrophoresis (SDS-PAGE) was performed on 0.25 μg protein. Pretreatment of 2.5 μg samples by incubation in 4 μL of 20 m*M* citrate, 1 m*M* ethylendiaminetetraacetic acid, pH 9.3 overnight at 4 °C was followed by 1:10 dilution in sample buffer (preincubation buffer with 1% (w/v) dithiothreitol and 3% (w/v) sodium dodecyl sulfate). Samples were heated at 100 °C for 8 min, and 4 μL/well was applied to a 20% T discontinuous gel (PhastSystem, Pharmacia Biotech, Uppsala, Sweden). Gels were developed by silver stain (PhastSystem sensitive stain protocol for SDS-PAGE).

Isoelectric focusing (IEF) of the same extracts used for SDS-PAGE were diluted in deionized water and IEF was performed on 0.1 μg of protein using a 3-9 PhastGel at 15 °C on a PhastSystem electrophoresis unit. Calibration standards were from the broad pI kit, pH 3-10 (Pharmacia). Silver stain (PhastSystem protocol) was used to reveal the focused samples and standards. The pI of protein extracted from AMF was determined by a calibration curve using the pI of standards vs distance of migration from the cathode.

Results and discussion

Antibody

The monoclonal antibody 32B11 was developed from spores of *G. intraradices* FL208 during an attempt to produce a specific antibody to identify the immunogen as had been done for *Glomus occultum* (Wright et al., 1987). Hybridoma 32B11 cross-reacted with freshly collected spores of other AMF in preliminary tests (data not shown), and was saved because of this characteristic.

Early IF tests with this antibody gave inconsistent results with spores and hyphae of AMF when tested as mature cultures or samples that had been dried for varying amount of time after being collected (data not shown). Only after testing fresh, active cultures of AMF was the potential revealed for use of MAb 32B11 to detect material on the surface of hyphae and some spores.

Friese and Allen (1991) and Wilson et al. (1983) produced polyclonal antibodies against *Gigaspora* spp. using hyphae from germinating spores as the immunogen. The antiserum of Friese and Allen (1991) had low specificity, but was useful to distinguish hyphae of an introduced *Gigaspora* isolate from native fungal hyphae. The antiserum of Wilson et al. (1983) detected material located inside hyphal walls or on broken ends of hyphae of the targeted species and also material on hyphae of an *Acaulospora laevis* isolate. Hyphae of *Glomus versiforme* was detected by antiserum produced against sporocarps (Kough and Linderman, 1986). The ubiquity of the antigen detected by MAb 32B11 poses the question as to how the above investigators were able to produce even moderately specific antisera to hyphae. It is possible that germinating spores do not produce a sufficient amount of the IM detected by MAb 32B11, or the protein is too weakly immunogenic to stimulate production of a dominant antibody in a polyclonal antiserum. Thin hyphae of germinating spores of *G. gigantea* MA453A have MAb 32B11-reactive material (not shown). However, the predominant hyphae from these spores was thick and melanized. Sporocarps used as the source of antigen by Kough and Linderman (1986) were dried at one stage of processing but probably retained at least some of the hyphal material described herein. However, the amount and condition of what remained may not have been sufficiently antigenic to stimulate a dominant antibody in a polyclonal antiserum. Indirect assays using rabbit antisera obtained by immunizations over greater than a few weeks using Freund's adjuvant (Kough and Linderman, 1986; Wilson et al., 1983) are probably predominantly composed of IgG molecules (Warr, 1982). The IgG molecule has two heavy chains for possible attachment of FITC-labeled anti-rabbit immunoglobulin probes. MAb 32B11, an IgM molecule, has 10 heavy chains (μ-chains) for possible attachment of FITC-labeled probes. MAb 32B11 could have magnified the FITC signal compared with

the supposed IgG-based assays. Thus, rabbit antiserum of previous workers may have contained low concentrations of antibodies against the ubiquitous hyphal protein detected by our MAb, but the reaction was too weak to detect. Also, previous reports of detection of AMF hyphae by using immunofluorescence do not indicate the biochemical nature of the antigen(s) for more detailed comparison with the material detected by MAb 32B11.

Characteristics of immunoreactive material on hyphae and plants

The IF assay used in this study provided the experimental tool to follow the appearance, distribution, and loss of an extracellular matrix on fungi in Glomales, and the appearance of a reactive material on colonized roots. The IM is synthesized on hyphae of all AMF tested, but the amount and distribution appears to vary among genera.

All isolates tested produced abundant hyphae and sporulated during three to four months of active plant growth of corn or Sudangrass in sand cultures. Figure 1A shows the nature and variation in thickness of the coating commonly seen on hyphae of *G. intraradices* UT126. In Figure 1B the continuity of IM on hyphae is shown. A freshly isolated spore of *G. intraradices* UT126 had a continuous layer of IM (Fig. 1C). A comparison with a photomicrograph of the same spore using incandescent light is shown (Fig. 1D). Some spores of this fungus examined later in the colonization process and some spores of other *Glomus* spp. had patches of IM. This may be the evanescent layer on spores described by Morton and Benney (1990) and was the likely source of the antigen which stimulated MAb 32B11.

Immunoreactive material on roots appeared as raised spots on the root surface (Fig. 1E). On root hairs IM varied from the spots described above on roots to a thick coating covering the tip of a root hair (Fig. 1F). Autofluorescence of roots generally appeared yellow, but occasionally root hairs had a homogeneous green fluorescence that did not extend beyond the structural boundaries of individual cells and was easily differentiated from IM. Colonization of Sudangrass and red clover was identical to corn in the appearance of IM on hyphae and roots. However, Sudangrass roots and root hairs had a greener autofluorescence than corn. Mycorrhizal roots tested with a *Rhizobium*-specific IgM MAb in place of MAb 32B11 or with FITC-labeled secondary antibody alone showed no IF. Uninoculated control roots of corn, Sudangrass, and red clover were nonreactive except for rare instances of fluorescent spots found in localized patches on root hairs similar to the smallest fluorescent spots seen in Figure 1F.

Descriptions of IM on hyphae and plants for isolates from the five AM fungal genera tested during active and late colonization are given in Table 1. After pot cultures were dried, IM on hyphae and roots declined at different rates for isolates tested but was greatly reduced to absent after 23-26 weeks.

The absence of IM on highly melanized hyphae may be due to melanin-inhibition of antibody binding (Cole et al., 1991). Extraction of hyphal protein from an isolate which produces abundant melanized hyphae such as *Glomus desterticola* Trappe et al., or electronmicroscopic studies of antibody attachment to melanized hyphae will be used in future studies to show whether attachment of the MAb is inhibited by melanin.

The IM detected by MAb 32B11 on the surface of hyphae of *Glomus* and thin hyphae of other genera resembles extracellular material on hyphal walls of many other fungi (Cole et al., 1973; Dowsett and Reid, 1981; Evans and Stempen, 1980; Evans et al., 1981; Palmer et al., 1983a, b). Immunofluorescent spots frequently seen on older hyphae of *Gigaspora* spp. (Fig. 1G) resemble cell wall excrescences numerous on older hyphae of *Bipolaris maydis* (Evans and Stempen, 1980). Similar dome-shaped spots of IM also were seen on mycorrhizal roots (Fig. 1E).

Several hypotheses are suggested to explain IM on plant as well as AMF hyphal parts: (i) IM originating from hyphae is deposited on roots during mycorrhizal development, (ii) plants are stimulated to produce the same IM as the hyphae, and (iii) plants produce a different molecule which shares the same antibody-specific epitope as the fungi. The first hypothesis is supported by spatial and environmental conditions in the region of root and hyphal growth which could provide a means of transfer of IM from fungal hyphae to root surfaces. Unattached IM particles often were seen on IF slide preparations and on IF of the plastic mesh used to hold sand in the bottom of pots (not shown) indicating that "peeling" and transfer of this material could occur. However, IM coating on root hairs appears to have been deposited in a more fluid state than the unattached particles seen on slide preparations, a factor that may be related to the age of the IM. Regarding the second hypothesis, it is known that unique molecules can be found in plants due to mycorrhizal development. Garcia-Garrido et al. (1993) isolated such polypeptides

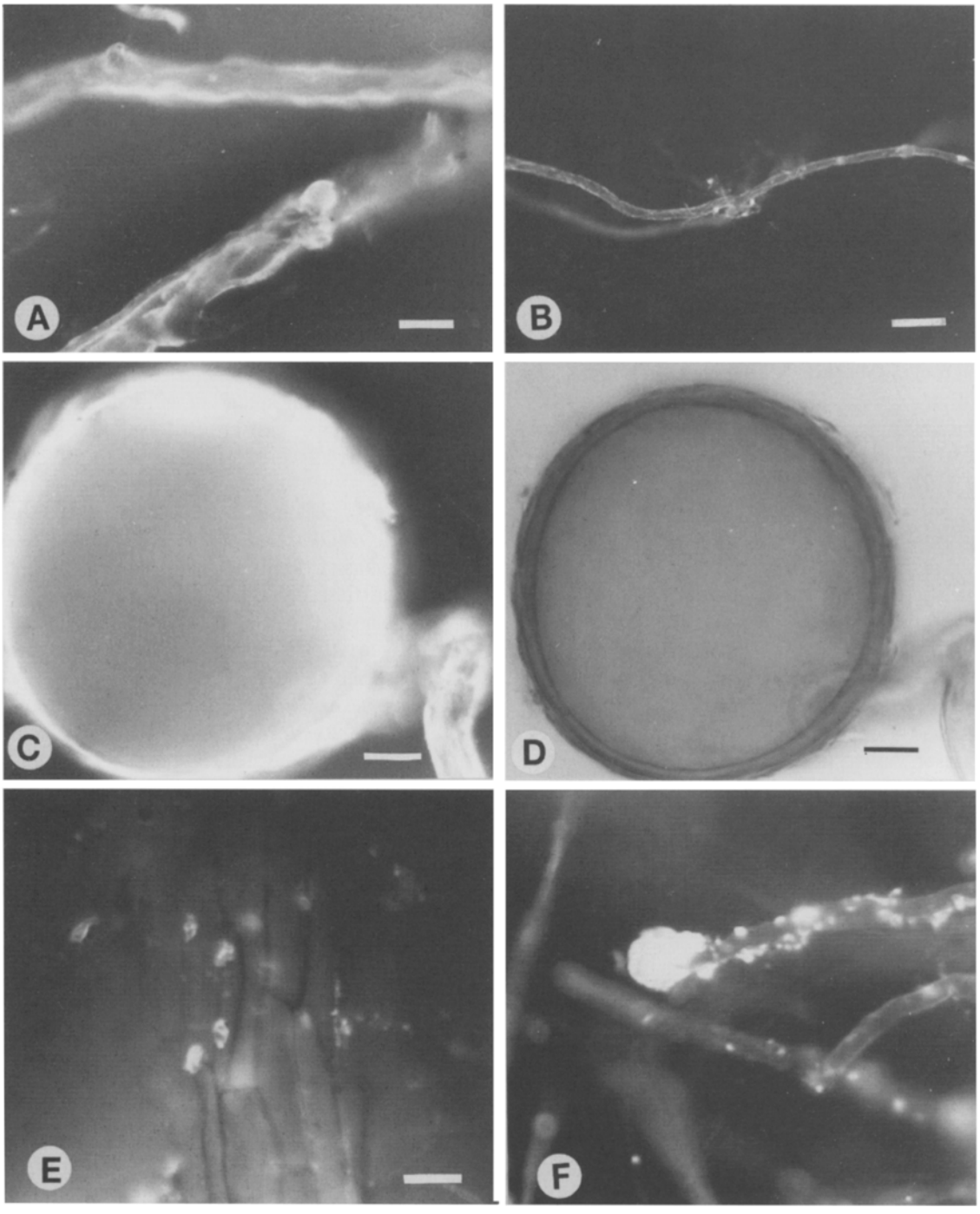

Figure 1. Photomicrographs showing immunofluorescent material (IM) detected by monodonal antibody 32B11 on fungal hyphae and plant roots. (**A** and **B**) IM is shown on hyphae of *Glomus intraradices* UT126 at 16 weeks after germination of the host plant Sudangrass (A, *Bar* = 6 μm; B, *Bar* = 60 μm). (**C** and **D**) IM on a spore of *G. intraradices* UT126 compared to the visible light view of the same spore (*Bar* = 15 μm). (**E**) Typical appearance of IM on roots as shown on Sudangrass colonized by *G. intraradices* UT126 (*Bar* = 15 μm). (**F**) IM on a root hair of Sudangrass colonized by *Acaulospora mellea* CL551. The smaller spots were typical of all AMF and all plant hosts studied but the mass of IM on the root tip was seen only on roots colonized by *A. mellea* (*Bar* = 60 μm). (**G**) IM on a hyphal strand of *Gigaspora rosea* UT102 showing domelike structures often seen on older cultures of this genus (*Bar* = 15 μm). (**H**) IM on hyphae of *Leptospharia korrea* (*Bar* = 60 μm).

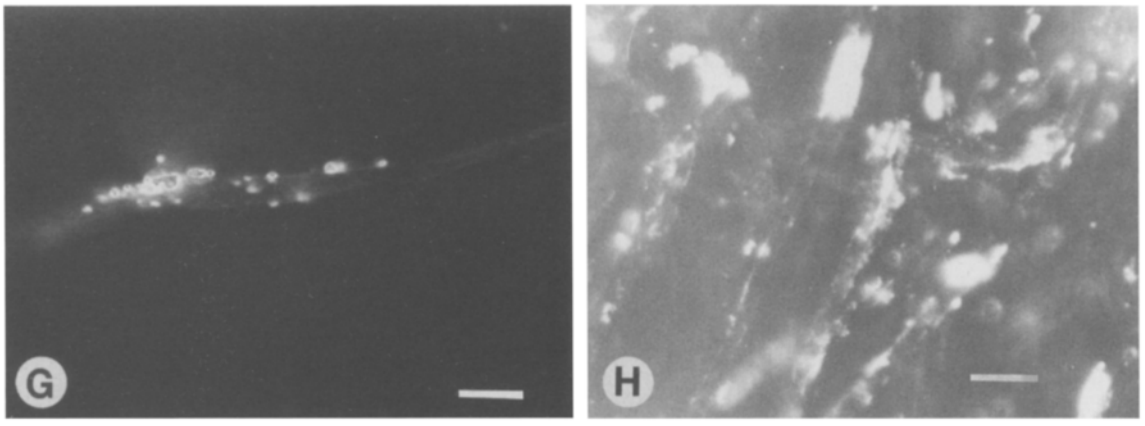

Figure 1. Fig. 1 contd.

Table 1. Time-course description of immunoreactive material (IM) on hyphae [H], plant roots [P], and spores [S] seen during colonization of corn by isolates from five genera of arbuscular mycorrhizal fungi

Genus	Active colonization (approx. 4-18 weeks[b])	Late colonization (approx. 20-22 weeks[b])
Glomus	[H] Thick brightly fluorescent layer with intermittent coarse aggregates weeks 8 to 13 (Fig. 1A and B). [P] Numerous localized spots[c] [S] Continuous to patchy layer surrounded new spores of some species (Fig. 1C and D).	[H] Brightness and thickness declined. [P] Numerous localized spots.
Acaulospora	[H] Irregular patches of IM; maximum brightness after week 11. [P] Early appearance of the largest amount of material seen for all isolates tested; material coated the tips some root hairs (Fig. 1F); spots on root surface.	[H] Generally the same as during active colonization. [P] Root hair coatings not detected; spots on roots and root hairs.
Gigaspora	[H] Thin hyphae (approx. 2μm) had a bright, continuous layer of material; thick, melanized hyphae (approx. 8 μm) had only rare patches. [P] Spots on roots and root hairs. [P] Root hair coatings on some species; spots on root hairs and root surface.	[H] Dome-shaped material (Fig. 1G) occurred on thin hyphae
Scutellospora	[H] Thin and thick hyphae; less thin hyphae than *Gigaspora* but the appearance of the material was similar; thin hyphae had a bright, continuous later; thick, melanized hyphae had granular patches of IM. [P] Spots on roots and root hairs.	[H] and [P] Less than during active phase.
Entrophospora	[H] Irregular patches of strongly fluorescent material. [P] Spots, but fewer than on other genera tested.	[H] and [P] Slightly less than during active phase.

[a]The following cultures were used to compile descriptions for genera: *Glomus intraradices* UT126 and FL208, and *G. etunicatum* UT316; *Acaulospora mellea* CL551; *Gigaspora rose* UT102; *G. gigantea* MA453A, and *G. albida* FL185; *Scutellospora heterogama* BR154C; *Entrophospora infrequens* (local isolate from Hagerstown, MD).
[b]Time after cultures were started.
[c]See Figure 1E for this and all other references in the table to immunoreactive spots on roots or root hairs.

from in vitro translation of total root RNA derived from *G. mosseae*-colonized onion roots. It is therefore possible that, by a similar mechanism, roots may be stimulated to produce the IM detected by MAb 32B11. The third hypothesis, cross-reactivity as a result of shared moieties on different molecules, remains a possibility until the reactive molecule is fully characterized (Harrison et al., 1994) and more is known about the first hypothesis.

Some non-AMF produced small amounts of IM on hyphae. In general the appearance of the IM was unlike the material detected on Glomales. *E. pisiformis* showed very slight reactivity only on the surface of spores. *R. solani*, *G. incrustrans*, *R. oligosporus*, *M. mucedo*, and *S. racemosum* isolates showed rare highly localized IM on hyphae. *Z. heterogamus* was generally non-reactive except for diffuse IM on very broad hyphae which was relatively rare in the hyphal mat. Hyphae of 33-day-old *G. virens* produced rare patches of faint and diffuse IM on hyphae. Relative to AMF, a large amount of fungal biomass was required to reveal IM on these fungi. *G. graminis* var. *avenae* and *P. megasperma* var. *glycinea* isolates were nonreactive. *L. korrea* was the most cross-reactive of non-AMF tested, but also required large quantities of fungal biomass to be detectable by IF. Hyphal mats from agar or broth cultures after 8 to 13 days of growth produced two hyphal morphotypes. Thicker hyphae within a hyphal mat showed patches of IM (Fig. 1H), and thinner hyphae had much less IM.

Extraction and characterization of immunoreactive material

Attempts were made to determine whether the reactive site of IM on hyphae was a polysaccharide or a protein. It became apparent that the native antigen was not easily solubilized and was resistant to chemicals often used to characterize or extract antigenic material. IF was not diminished on hyphae exposed to acidic or basic solutions or to heat, with the exception of citric acid (pH 6.0) at 100 °C where *G. gigantea* MA453A showed reduced IF compared with the control. The native antigen also was resistant to extraction with hot SDS. A polysaccharide was suggested by heat resistance. However, we were unable to determine whether the polysaccharide was attached to a protein because of apparent resistance of the native molecule to digestion by pronase, trypsin, and endoglycosidase-H as assessed by IF (not shown).

The resistance of the antigen to common extraction procedures and to heat led to the use of a protocol suggested by Keen and Legrand (1980) which required autoclaving in citrate buffer. Results of protein assays and ELISA on extracts are given in Table 2. Results for *G. gigantea* MA453A are from TCA precipitated protein which indicate that MAb 32B11 reacted with a site on a protein or glycoprotein rather than with an unattached carbohydrate. Also, protein extracted from *L. korrea* hyphae was compared with AMF protein. Controls for the ELISA were non-reactive.

The amount of protein extracted per mg of hyphae varied among genera and between species tested (Table 2). Slight variations in protein yield for an isolate were suggested by the variation in protein from *G. intraradices* UT126 hyphae in two different pot cultures. Weight of the air-dried hyphae of *G. etunicatium* UT316 included a large number of trapped spores which may have led to the low calculated protein yield. Further work is necessary to more accurately define differences among genera and isolates, but these results suggest that *Gigaspora* spp. may produce more protein/mg of hyphae than *Glomus* spp. Comparative tests on protein extracted from *L. korrea* indicate that the protein yield from this fungus was orders of magnitude less than for AMF and the protein was less reactive with MAb 32B11. ELISA reactions of the same amount of protein from different AMF genera and isolates suggests that the reactive site on freshly extracted protein does not vary greatly (Table 2). The results presented here indicate that AMF are the primary producers of the protein detected by the MAb.

Lectin binding is a general test for a glycoprotein. Moderate to strong binding of freshly extracted protein to ConA, SBA, and WGA and weak binding to LcH was indicated for the isolates tested (Table 3). ConA binds to D-mannopyranose or D-glucopyranose; SBA binds to D-galactose or N-acetyl-D-galactosamine residues; WGA binds N,N′-diacetylchitobiose and N,N′,N′-triacetylchitotriose; and LcH reacts with D-mannopyranose, but less stongly than ConA. Relatively lower binding of LcH compared with ConA to the fresh samples tested was expected from the general properties of these lectins. Protein from *G. gigantea* MA453A which had been stored over 6 months had apparently lost reactivity with ConA and WGA, but long-term storage of the protein from *G. intraradices* FL208 apparently did not result in such a substantial loss of reactivity. The possibility that IM contained lectin-reactive carbohydrates linked to a protein was suggested by the procedure necessary to extract IM.

Table 2. Comparison of yield and immunoreactivity of protein extracted from hyphae of arbuscular mycorrhizal fungi (AMF) and a non-AMF fungus reactive with monoclonal antibody 32B11

Culture	Hyphae wt. (mg)	Total protein (μg)	Yield ($\mu g\ mg^{-1}$)	ELISA[a] A_{405}
Gigaspora gigantea MA453A	2.4	152	63	1.473±0.031[b]
Gigaspora rosea UT102	1.3	79	60	1.294±0.045
Glomus etunicatum UT316	5.1	61	12	1.295±0.030
Glomus intraradices UT126[c]	1.2	35	21	1.179±0.042
Glomus intraradices UT126[c]	3.2	67	29	ND[d]
Glomus intraradices FL208	44.6	767	17	ND
Leptospharia korrea	180	9	0.05	0.242±0.038

[a]Enzyme-linked immunosorbent assays. Results represent mean and SD for readings on four replicates of 0.5 μg of protein.
[b]Protein extracted from *G. gigantea* MA453 was precipitated and washed before the the protein was diluted for ELISA. All other ELISA results are from dilutions of crude extracts.
[c]Two separate extractions from different pot cultures.
[d]Not determined.

Table 3. Binding of biotinylated lectins to extracted protein from various isolates of arbuscular-mycorrhizal fungi detected by streptavidin-peroxidase using a microtiter plate format

Isolate	A_{405} ConA[a]	SBA	LcH	WGA
Gigaspora gigantea MA453A[b]	0.430(±0.042)[d]	0.413(±0.062)	0.141(±0.020)	0.461(±0.098)
Gigaspora gigantea MA453A[c]	0.083(±0.017)	0.444(±0.030)	0.130(±0.006)	0.115(±0.023)
Gigaspora rosea UT102[b]	0.761(±0.119)	0.656(±0.013)	0.255(±0.093)	0.797(±0.031)
Glomus intraradices UT126[b]	0.662(±0.020)	0.451(±0.026)	0.204(±0.027)	0.496(±0.061)
Glomus intraradices FL208[b]	0.881(±0.122)	0.727(±0.053)	0.217(±0.038)	0.689(±0.044)
Glomus intraradices FL208[c]	0.717(±0.040)	0.614(±0.048)	0.188(±0.022)	0.496(±0.024)
Glomus etunicatum UT316[b]	0.899(±0.135)	0.520(±0.039)	0.190(±0.030)	0.393(±0.070)

[a]ConA = succinyl concanavalin A, SBA = *Glycine max* agglutinin, LcH = *Lens culinaris* agglutinin, and WGA = *Triticum vulgaris* agglutinin; 2.5 μg of lectin per microtiter plate well was used for each.
[b]Extract was < 1 month old at the time it was tested and had been filter sterilized and stored in water.
[c]Extract was > 3 months old at the time it was tested and had been filter sterilized and stored in water.
[d]Mean and SD of three replicates.

Mannan, a major mannose polysaccharide of yeast, is separated from the cell-wall matrix by autoclaving in a neutral citrate buffer and contains covalently-linked protein or polypeptide, D-galactose and N-acetyl-D-glucosamine residues (Ballou, 1976). Glycosylation of AMF hyphal protein would explain the thermal stability, insolubility, and resistance to proteolysis of this molecule (Bahl, 1992). Further work is in progress to confirm that the molecule is a glycoprotein.

IEF and SDS profiles as determined by the described methods indicated marked similarity among protein extracted from *Gigaspora* and *Glomus* isolates. The focused native proteins on IEF 3-9 gel from all isolates tested showed two distict bands: a broad band between pH 7.3 and 7.5 and a minor band at pH 6.9 (Fig. 2). SDS-PAGE of extracts for the four isolates revealed the bands shown in Figure 2. Denaturing of the protein depended on the ratio of protein to each ingredient in the sample buffer. When the conditions were not precise, only a smear was seen. As the protocol was being developed, a doublet and a more rapidly migrating single band were consistently seen between the 94 and 67 Kilodalton markers. Preincubation of the protein overnight at a high pH was required to accomplish the denaturing process (Fig. 2A). The denatured protein was not sufficiently reactive with the antibody to detect immunoreactive bands after transfer from SDS gels to a membrane. Exact molecular

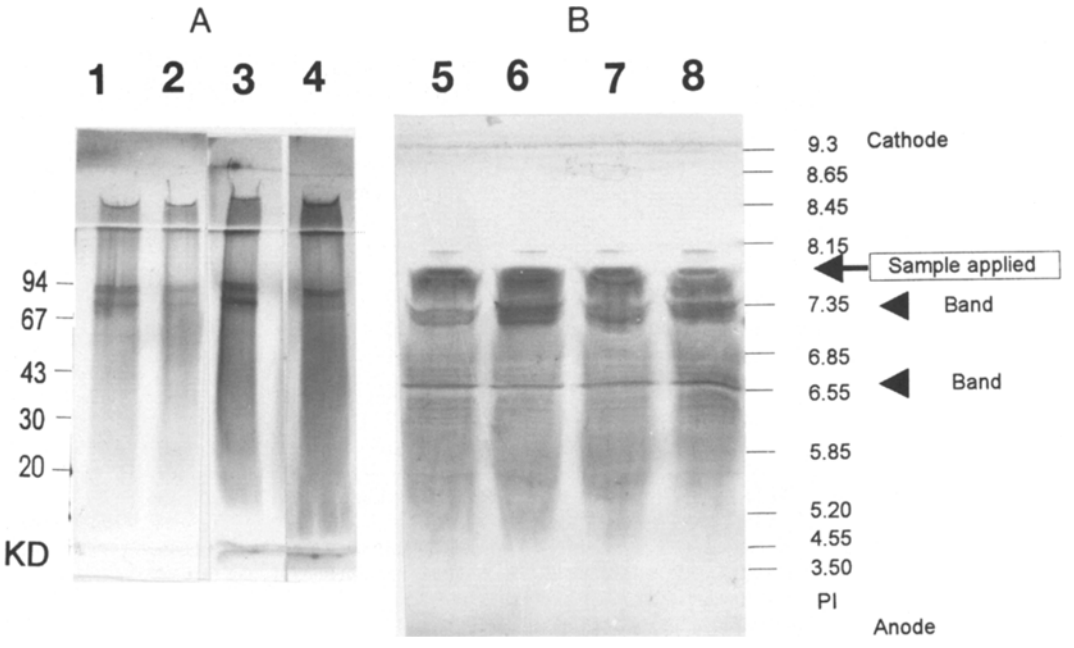

Figure 2. SDS-PAGE (**A**) and isoelectric focusing (**B**) gels of protein extracted from hyphae of four species of arbuscular mycorrhizal fungi. Both types of gel were developed by silver stain. *Gigaspora gigantea* MA453 A in Lanes 1 and 5; *Glomus intraradices* UT126 in Lanes 2 and 6; *Gigaspora rosea* UT102 in lanes 3, and 7; and *Glomus intraradices* FL208 in lanes 4 and 8. Position of molecular weight markers for the SDS-PAGE gel are shown on the left. Positions of standards used to generate a curve for pH vs distance of migration from the cathode are shown on the right.

weights of bands on SDS gels and a pI for IEF gel bands were not assigned because of the putative glycoprotein nature of IM (Carlsson, 1993; Righetti and Drysdale, 1976). However, these results indicate that the proteins extracted from different isolates are alike when treated by the conditions used to obtain these profiles.

Further work is necessary to reveal how the protein ages both in the native state and after extraction, and why it is so resistant to solubilization and denaturation. Extracted protein from a *Glomus* isolate and a *Gigaspora* isolate showed about a 10% loss due apparently to precipitation after storage at −20 °C, and 24 h incubation at 4 °C at pH 4.5 caused almost total degradation (data not shown). Protein extracts from hyphae stored in 20 m*M* pH 7.0 citrate buffer at 4 °C for several weeks showed no, or only slight, degradation as determined by both protein assay and ELISA and did not readily support the growth of air-borne contaminants. However, longer storage has resulted in loss of some of the characteristic reactions of fresh extracts (Table 2). Extracts stored in non-sterile water rapidly became contaminated with bacteria and were degraded. MAb 32B11 will be standardized by ELISA on freshly extracted protein from hyphae of *Glomus* or *Gigaspora* harvested during active colonization.

Hydrophobicity of IM would explain adhesion to plastic mesh in pot cultures (Rosenberg and Doyle, 1990) and may explain adhesion to plant surfaces and (Smit and Stacey, 1990). However, the putative glycoprotein nature of AMF hyphal protein is not characteristic of the class of proteins called hydrophobins (De Vries et al., 1993; Doyle and Rosenberg, 1990; Wessels, 1993; Wessels, et al., 1991).

Loss of IM from hyphae upon drying of pot cultures, and the harsh conditions required to extract the protein from fresh hyphae led us to explore the possibility that this protein could be found in soil long after AMF cease active colonization. We are able to extract large amounts of a protein from dry soils using the extraction procedure described herein for hyphae. Yields of protein range from 4.4 to 14.4 μg protein mg^{-1} soil in A horizons of 12 different soils collected from the eastern United States. Analysis by ELISA, SDS-PAGE and IEF indicate that the soil protein has very similar characteristics to those described for the AMF protein (manuscript in preparation). Also, the possibility that AMF contribute a water-stable protein

to soil aggregates can be shown by the presence of IM on aggregate surfaces (manuscript in preparation).

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